

Chapter 28

Multicellular Eicosanoid and Other Metabolic Interactions of Platelets and Other Cells

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Lipids, carbohydrates, and proteins constitute the major structural components of living cells. A lipid may be defined as a biomolecule that is insoluble in water but soluble in low-polarity solvents such as chloroform, ether, and ethanol. The latter are commonly used to extract lipids from cells and tissues. This definition is broad because it includes substances such as steroids and terpenes.¹ Fats are a subdivision in the lipid class and represent the main storage form of lipids in cells. In liquid form, fats are classified as oils (corn oil, coconut oil, and so forth). Fats are carboxylic esters derived from glycerol and are also known as triglycerides. Fats are stored in specific cells in plants and animals and when oxidized provide energy for basic biologic processes.

Phospholipids (phosphoglycerides) are the most important functional lipids in platelets and other cells. In contrast to triacylglycerols, phospholipids contain two acyl groups and a phosphate group esterified to the third carbon. Thus, the "parent structure" of phosphoglycerides is phosphatidic acid (diacylglycerol phosphate). Phospholipids are components of all cell membranes and represent the major structural components of the living cell. The physical properties of phospholipids are unique, and this accounts for their critical role in cell function. The fatty acid portions of phospholipids are lipophilic, and the dipolar ionic portions are hydrophilic. The substituted phosphate group at the dipolar end can be positively or negatively charged. In purely aqueous solutions, phospholipids exist in the form of micelles. However, at the interface between two different aqueous solutions, phospholipids form bilayers, with two rows of molecules facing each other and the polar portion projecting into water on the two surfaces of the bilayer.¹

Phospholipids exist in cell membranes as bilayers and form a mechanical and biochemically functional barrier between the cell and the external milieu. Passage of nutrients, waste products, and other substances of high polarity is controlled at the cell surface. This selectivity resides in proteins that are embedded in the phospholipid bilayer. Thus, phospholipids and lipids in general rarely exist in cells or tissues in free form. They usually are found in a macromo-

lecular array with protein or carbohydrates (lipoproteins or lipopolysaccharides). In the case of platelets, lipoprotein "rearrangements" take place on the surface when they are activated. This gives rise to the unique ability of platelet phospholipoproteins to promote coagulation.²

Physical or chemical perturbation of platelets, endothelial cells, or neutrophils results in immediate release of free arachidonate from phospholipids. The arachidonic acid is then enzymatically oxygenated and subsequently converted to biologically active compounds known as eicosanoids. This term includes the "classical" prostaglandins, the thromboxanes, prostacyclin, and hydroxy acids such as leukotrienes. Eicosanoid intermediates and end products not only serve as autacoids that act on neighboring cells and tissues in the microenvironment, but also participate in biochemical cell-cell interactions (one example is transcellular metabolism).³

Platelet lipid and eicosanoid research has evolved through three main phases since 1955 and is now entering a fourth. The initial *analytical* phase spanned approximately 13 years, from 1955 through 1968. The *metabolic* phase extended from 1969 through 1973, and the *functional* phase, which actually developed during the preceding two phases, began in 1974 and extends into the 1990s.⁴⁻⁷

A fourth phase has started that will extend into the 1990s and will have two main components: (1) studies of transcellular metabolism of eicosanoids and (2) molecular biological studies of enzymes catalyzing eicosanoid formation.⁸⁻¹⁰ These studies deal with the fact that different cell types are capable of metabolizing eicosanoid precursors and intermediates into metabolites that neither cell can produce alone, and that two cells can produce markedly increased quantities of a metabolite compared with the amount that either cell can synthesize alone.^{11,12}

In the early 1950s, silicic acid paper and column chromatography rendered it possible to separate and identify individual lipid classes from tissues or cells such as platelets and endothelium. Gas-liquid chromatography (GLC) permitted identification of fatty acids and aldehydes in a specific lipid class. The ability to carry out GLC on small samples was uniquely advantageous for hematologic cells.

TABLE 28-1 Platelet Lipid Miscellany

500 ml of whole blood contains 1.5–2 g platelets (wet weight).
 1 U of platelet-rich plasma contains 10^{11} platelets.
 Water content of platelets = 87%.
 1 g (wet weight) of platelets contains 31 mg of total lipid.
 10^{11} platelets contain 146 mg of protein.
 10^{11} platelets contain 13 mg of membrane protein.
 10^{11} platelets contain 8 mg of membrane lipid.
 N-Acetyl neuraminic acid (NANA) content of platelets is 8 $\mu\text{g}/\text{mg}$ protein.
 6% of platelet NANA is present in the ganglioside fraction.
 A "thrombocrit" of 1% represents 10^9 platelets/ml.
 Total volume of circulating platelets in man is about 10 ml–15 ml.
 Lipid/protein ratio (w/w) in whole platelets = 0.28.
 Lipid/protein ratio (w/w) in platelet membranes = 0.58.
 Phospholipid/total lipid in whole platelets = 79%.
 Phospholipid/total lipid in platelet membranes = 78%.
 Molar ratio of cholesterol to phospholipid in platelet membranes = 0.53.

Thin-layer chromatography (TLC) permitted qualitative and quantitative analytical studies of lipids in blood components. Samples could be eluted from TLC plates and examined further by high-performance liquid chromatography (HPLC). This has been the methodologic basis for biochemical studies of hydroxy acids such as leukotrienes and lipoxins.^{13,14} Mass spectroscopy has provided detailed structural analysis of virtually all eicosanoids.^{15,16} Information on lipid and protein parameters in platelets and endothelial cells is summarized in Tables 28-1 through 28-3.

A major aspect of vascular research is arachidonic acid metabolism. Arachidonic acid is a polyunsaturated fatty acid synthesized in mammalian cells from linoleic acid in the diet. Arachidonic, linoleic, and linolenic acids are known as essential fatty acids (ie, they cannot be synthesized *de novo* by higher mammalian species). Diets devoid of essential fatty acids will induce a metabolic disturbance known as *essential fatty acid deficiency*.

Polyunsaturated acids were crucial to the evolutionary development of cell membranes. In plants, linolenic acid is the major fatty acid in glycolipids of chloroplast membranes and is required for function of photosynthetic units. In mammals, arachidonic acid is important for structural and functional integrity of cell membranes and is the precursor of all eicosanoids. The term *eicosanoid* defines all oxygenated derivatives of arachidonate: the derivatives contain 20 carbon atoms. The term *prostaglandin* refers to one group of eicosanoids.³

TABLE 28-2 Human Platelet Lipids

Lipid Class	Weight (%)
"Neutral" lipids	21
Phospholipid	77
Ganglioside	0.5
Proteolipid	1.8

(Data from Marcus AJ, Uilman HL, Safier LB: Lipid composition of subcellular particles of human blood platelets. *J Lipid Res* 10:108, 1969)

TABLE 28-3 Phospholipid Composition of Endothelial Cells and Platelets*

Phospholipid	Endothelial Cells†	Platelets
Phosphatidic acid (PA)	Trace	Trace
Phosphatidylethanolamine (PE)	25.7	28.1
Lysophosphatidylethanolamine (LPE)	Trace	Trace
Phosphatidylserine (PS)	7.2	10.5
Phosphatidylinositol (PI)	6.3	4.6
Phosphatidylcholine (PC)	50.9	38.5
Lysophosphatidylcholine (LPC)	1.2	1.0
Sphingomyelin (Sph)	7.8	16.0

* Percentage of lipid phosphorus.

† Values represent the means of four different pools of endothelial cells.

The discovery that eicosanoids originated from arachidonic acid was a major advance in biomedical research.^{17,18} The structure of eicosanoids and related compounds is depicted in Figures 28-1 and 28-2. The polyunsaturated fatty acid in fish oils, eicosapentaenoic acid (20:5), is also a source of eicosanoids (eg, thromboxane A_2 , prostaglandin I_2 , and leukotriene B_5). They are structurally different than those from 20:4 (arachidonic acid). These omega-3-derived eicosanoids have been reported to possess antithrombotic and antiinflammatory properties.¹⁹

Eicosanoid Metabolism in Human Platelets and Endothelial Cells

As shown in Tables 28-4 and 28-5, arachidonate is a major fatty acid in platelets and endothelial cells. It is present in intracellular granules and membranes, principally esterified to the phospholipid moiety. In general, arachidonic acid cannot be oxygenated unless it is initially hydrolyzed from phospholipids by phospholipases and made available in "free" form.

Liberation of arachidonate from cell phospholipids occurs by way of two mechanisms. One involves the sequential action of a phosphatidylinositol-specific phospholipase C, followed by the action of a diglyceride lipase, yielding free arachidonate. In addition, there is a platelet phospholipase A_2 activity that liberates arachidonate from phosphatidylcholine and phosphatidylethanolamine. Platelets can also reacylate free arachidonate as well as release it to the extracellular milieu where it becomes available for uptake by other cells (transcellular metabolism).²⁰ The platelet phospholipase(s) systems are important for future research because a drug that would limit or completely block arachidonic acid hydrolysis could interfere with release of prothrombotic or proinflammatory eicosanoids.

Eicosanoids are not preformed or stored in cells. They are always newly synthesized in response to specific agonists or physical perturbation. Calcium is required for lip-

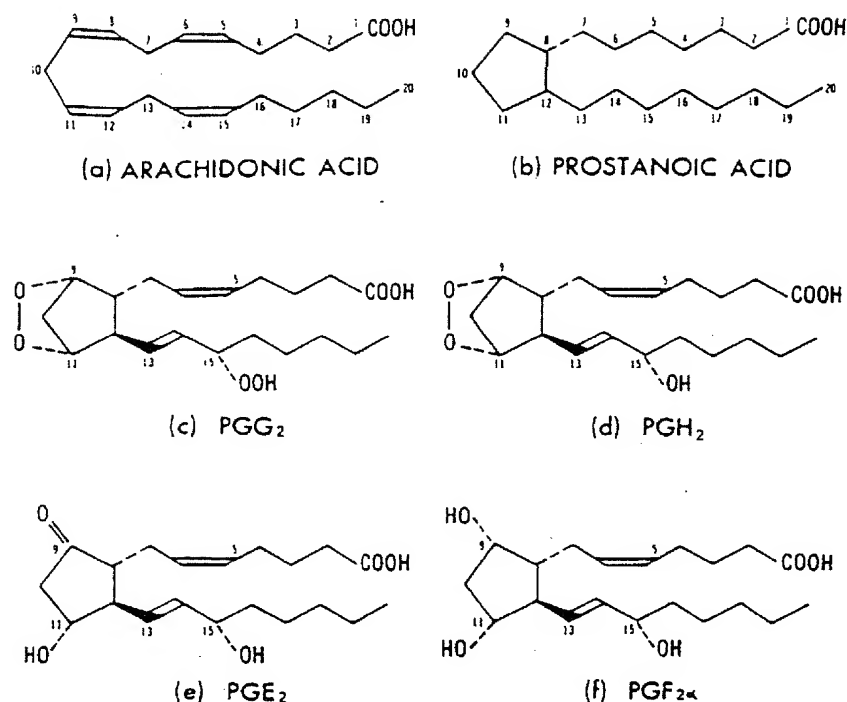


FIGURE 28-1 (A) All-*cis*-5,8,11,14-eicosatetraenoic acid (20:4). A major fatty acid in phospholipids of platelets and endothelial cells. After hydrolysis by phospholipase(s), cyclization to prostaglandins ensues. Mammals can synthesize saturated and monounsaturated fatty acids but cannot form linoleic or γ -linolenic acids. These must be obtained from the diet. Arachidonic, linoleic, and linolenic acids are essential fatty acids for mammals and are required for prostaglandin synthesis. Arachidonic acid is an important structural component of cells and is also the precursor for thromboxane formation in platelets and prostacyclin in endothelial cells. (B) The theoretic precursor of prostaglandins is prostanoic acid. This is a fully saturated C₂₀ acid with a cyclopentane ring that forms by closure from C₈ to C₁₂. (C) 15-Hydroperoxy-9 α , 11 α -peroxidoprostano-5, 13-dienoic acid (prostaglandin G₂). This is the initial oxygenated compound formed from 20:4. The reaction is catalyzed by cyclooxygenase. Oxygenation occurs at C₁₁ and C₁₅. An oxygen radical is present on C₁₁ that attacks C₉ to form a peroxide bridge. Cyclooxygenase preparations contain peroxidase activity; thus, PGG₂ may have a short life span in vivo and is rapidly reduced to PGH₂. (D) 15-Hydroxy-9 α , 11 α -peroxidoprostano-5,13-dienoic acid (prostaglandin H₂). This is a more polar compound than PGG₂ and in aqueous media yields PGE₂. (E) Prostaglandin E₂. This is a major prostaglandin produced in many tissues, including endothelial cells and kidney. (F) PGF_{2 α} . No specific function has been attributed to this prostaglandin, which is formed in endothelial cells.

ase activation, but it is still not completely clear how phospholipases initially become activated. Collagen, thrombin, and ionophore are the most active eicosanoid-eliciting agonists of platelets and endothelial cells. Platelet eicosanoid production is less stimulated by adenosine diphosphate (ADP) or platelet activating factor (PAF).

Released free arachidonate can leave the cell and become available for metabolic processing by a neighboring cell. When platelets and neutrophils are activated by a common stimulus, released platelet arachidonate is metabolized to leukotriene B₄ (LTB₄) by the activated neutrophil.²⁰ Furthermore, free arachidonate in extracellular fluids can be bound by plasma albumin, which serves as a "sink" or "reservoir" for arachidonate, which can then be used again.²¹

Formation of Arachidonic Acid Endoperoxides in Platelets

The PGG/H synthase system controls enzymatic cyclooxygenation of free arachidonate in platelets and endothelial cells. This synthase is a particulate enzyme located in the endoplasmic reticulum (in platelets, the dense tubular system). The PGG/H synthase system is heme dependent and substrate activated. It contains two activities, a bis-oxygenase (cyclooxygenase), which catalyzes PGG₂ formation from arachidonate, and a hydroperoxidase, which catalyzes a two-electron reduction of the 15-hydroperoxy group

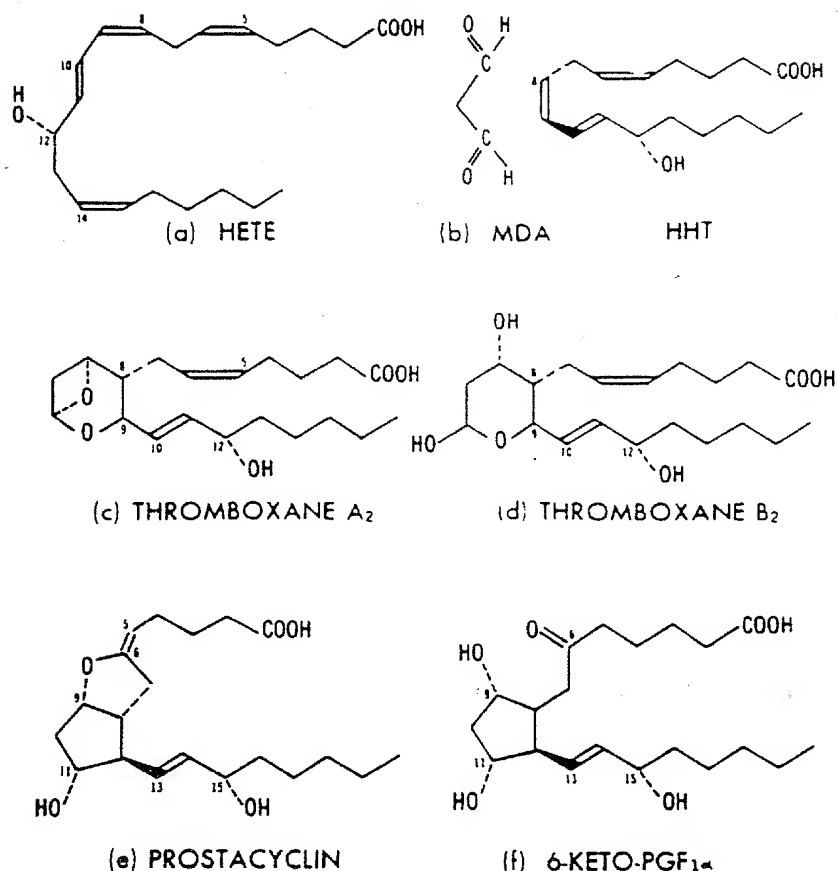


FIGURE 28-2 (A) 12-Hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE). This is the end product of the lipoxygenase pathway in human platelets. 12-HETE is formed by reduction of its precursor 12-hydroperoxy-5,8,10,14-eicosatetraenoic acid 12-HPETE. Production of 12-HETE is not inhibited by aspirin or indomethacin, and in fact it increases because of greater availability of free arachidonate. 12-HETE is metabolized by neutrophils and also enhances cholesteryl ester hydrolase activity in smooth muscle. (B) 12-Hydroxy-5,8,10-heptadecatrienoic acid (HHT; new terminology—HHTre). This compound forms as a by-product of the reduction of $\text{PGG}_2/\text{PGH}_2$. The endoperoxides are fragmented to a 17-carbon compound, accompanied by release of malondialdehyde. Nonsteroidal antiinflammatory agents also inhibit synthesis of HHTre from arachidonic acid in platelets. HHTre has no known biologic function. (C) Thromboxane A₂ (TXA₂) is an unstable intermediate ($t_{1/2} = 32$ seconds) in the pathway between $\text{PGG}_2/\text{PGH}_2$ and thromboxane B₂. TXA₂ induces approximately 28% serotonin release from platelets, which is relatively modest and comparable to that induced by ADP and PAF (platelet activating factor). TXA₂ is also a vasoconstrictor, which may add to or synergize with the vasoconstriction induced by released platelet serotonin.

of PGG_2 , resulting in PGH_2 . Enzymatic activity of cyclooxygenase is blocked by aspirin and other nonsteroidal antiinflammatory drugs. The biochemical steps described above are summarized in Figures 28-3 and 28-4.

The PGG_2/H synthase system is extremely important clinically because it is inhibited by aspirin. After aspirin ingestion, a serine residue of the cyclooxygenase becomes O-acetylated by the acetyl portion of the molecule. The serine moiety is located 70 amino acids from the C-terminus. This irreversible acetylation reaction was initially demonstrated when it was observed that platelets and subcellu-

lar platelet fractions were labeled by radioactive aspirin containing the label in the acetyl group. This was not the case with aspirin radiolabeled in the carboxyl group.²² Other nonsteroidal antiinflammatory agents such as indomethacin, meclofenamate, and ibuprofen can also inactivate the PGG_2/H synthase irreversibly, but not by way of covalent modification of the enzyme, as in the case of aspirin. Ibuprofen is a reversible inhibitor of cyclooxygenase and also competes with free arachidonate for cyclooxygenase binding. In some cell systems, the PGG_2/H synthase gene is regulated by the mitotic cycle.²³

TABLE 28-4 Phospholipid Fatty Acids and Aldehydes of Human Platelets (Weight %*)

	Phosphatidylethanolamine (PE)	Phosphatidylserine (PS)	Phosphatidylinositol (PI)	Lecithin (Lec)	Sphingomyelin (Sph)
16:0 DMA	9.2	0.60	0.92	0.80	
16:0	3.4	0.97	1.6	34.1	24.6
16:1	0.26	0.67	0.46	1.6	1.2
18:0 DMA	17.9	0.19	0.12	Tr.	
18:0	13.7	44.7	44.7	14.1	5.8
18:1 DMA	2.7				
18:1	5.9	26.7	8.7	27.0	1.3
18:2	2.3		0.05	6.9	
18:3	1.6				
20:0	0.64	1.6	0.84	0.38	9.4
20:4	31.8	22.6	41.8	12.0	Tr.
22:0					29.3
22:1					2.0
22:4 (n - 6)	4.4				
22:5	1.8	Tr.		Tr.	
22:6	1.2	Tr.			
23:0					1.2
24:0					10.9
24:1					12.8

Fatty acids are designated by chain length : number of double bonds. In addition to those shown, the following acids were detected in small (<1.5) percentages: 12:0, 14:0, 15:0, 17:0 DMA, 17:0, 20:1, 20:2, 20:3, and one with a probable carbon number of 22 on Apiezon. Aldehydes derived from plasmalogens are measured as dimethylacetals (DMAs).

Tr, trace.

* Determined by gas-liquid chromatography on ethylene glycol adipate polyester or Apiezon, or both.

(Data from Marcus AJ, Ullman HL, Safier LB: Lipid composition of subcellular particles of human blood platelets. *J Lipid Res* 10:108, 1969)

TABLE 28-5 Phospholipid Fatty Acids and Aldehydes of Cultured Human Endothelial Cells

	PC	PE	PS	PI	Sph
	C	C	C	C	C
14:0	0.96	0.8	1.1	0.8	1.1
16:0 DMA	1.0	10.4	0.4	0.3	
16:0	43.4	7.9	5.5	6.3	44.4
16:1	3.3	0.7	1.1	1.6	0.5
18:0 DMA	1.0	7.3	0.5	0.3	
18:1 DMA		2.2			
18:0	8.1	16.3	41.1	37.7	7.9
18:1	17.9	10.8	19.8	9.6	1.5
18:2	10.5	4.8	4.7	4.2	0.4
20:0					1.8
20:3	2.0	2.0	4.3	3.0	
20:4	6.5	19.8	7.1	33.3	
20:5		0.4	1.1		
22:0					8.3
22:4	1.2	8.8	6.4	2.6	
22:5	0.4	3.2	2.9	0.6	
22:6	0.6	5.0	3.2	0.5	
23:0					2.0
24:0					12.2
24:1					15.6
?					3.3

DMA, dimethylacetal(s).

Aspirin is probably the most commonly consumed pharmacologic agent in the world. Production in the United States alone is estimated at 43 million pounds per year. It remains difficult to comprehend the actions of aspirin as an analgesic, antipyretic, and antiinflammatory medication. Aspirin does not prevent production of leukotrienes—eicosanoids produced by the 5-lipoxygenase pathway (see Fig. 28-3). Leukotrienes are the most potent proinflammatory substances described. A "leukotriene paradox" has been posed: How can aspirin exert its antiinflammatory effect in the setting of abundant LTB₄ production?

The cyclooxygenase of the aspirin-treated platelet is permanently inactivated because platelets cannot synthesize appreciable new protein. Such platelets are unresponsive to arachidonic acid stimulation in vitro, and reactivity to other agonists such as ADP, thrombin, collagen, and epinephrine is reduced but not totally inhibited. The in vivo consequence of this defect is prolongation of the bleeding time, usually by 3 to 5 minutes. Such prolongation represents the antihemostatic effect of aspirin. This, however, may not be synonymous with an antithrombotic effect. Aspirin treatment does offer a degree of protection for patients with occlusive vascular diseases, based on cumulative results in several clinical trials.²⁴ The beneficial effects of aspirin in thrombotic diseases could be unrelated to or superimposed upon its inhibitory effect on platelet cyclooxygenase. The platelet lipoxygenase product 12-HETE is produced in abundance during aspirin administration.

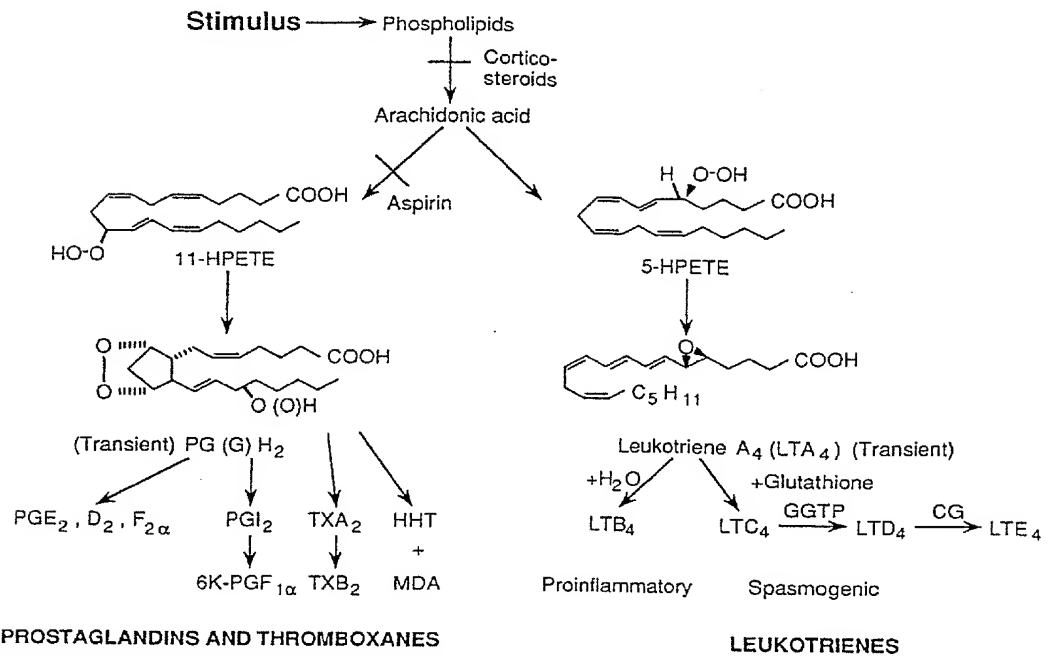


FIGURE 28-3 Biochemical reactions involved in eicosanoid formation from arachidonic acid. The first rate-limiting step is hydrolysis of esterified arachidonic acid from cell phospholipid. This can be blocked by corticosteroids. The cyclooxygenation step (upper left) is inhibitable by aspirin and results in formation of oxygenation products known as endoperoxides. Biochemical transformation of endoperoxides to other eicosanoids is cell specific and tissue specific. Thromboxane A₂ and PGI₂ are formed enzymatically and measured as their end products, thromboxane B₂ and 6-keto-PGF_{1α}. The 5-lipoxygenase pathway (upper right) exists in cells mainly involved in host defense mechanisms. Those containing a sulfur moiety at carbon 6 are spasmogenic (lower right). Leukotriene B₄ is a highly potent chemoattractant and chemokinetic eicosanoid. It is a matter of great interest that arachidonic acid can be converted into such a large variety of metabolic derivatives with highly divergent biologic activities. (Modified from Samuelsson B, Dahlen S-E, Lindgren JA et al: Leukotrienes and lipoxins: Structures, biosynthesis, and biological effects. *Science* 237:1171–1176, 1987)

This eicosanoid may play an antithrombotic role by itself or by conversion to another metabolite by way of transcellular metabolism involving such cells as neutrophils.^{3,25,26} Leukotriene production is reduced during transcellular metabolism of 12-HETE by neutrophils, which may partially explain the antiinflammatory effects of aspirin.

One of the most interesting aspects of eicosanoid metabolism is the diverse group of end products that result from PGH₂. The eicosanoid(s) subsequently synthesized depends on the particular enzymes present in the cell or tissue under study. For example, if the cell contains a PGH₂-PGD₂ isomerase, PGD₂ will form. This compound is a vasodilator produced in endothelial cells. The PGH₂/PGE₂ isomerase transforms PGH₂ to PGE₂. This eicosanoid is important in endothelial cells and in the kidney, where it plays a role in renal water resorption. In the kidney, reduced glutathione (GSH) is a required cofactor for PGE₂ synthesis. Whether this is true for endothelial cells has not been elucidated. If a cell contains the PGF_{2α} reductase, PGH₂ will be converted to PGF_{2α}. The latter is the major eicosanoid produced by uterine endometrium. In endothe-

lial cells, the prostacyclin synthase catalyzes the formation of prostacyclin (PGI₂) from PGH₂.²³

Thromboxane A₂ induces platelet aggregation accompanied by release of about 30% of the platelet content of serotonin. This is comparable to the release resulting from action of ADP or platelet activating factor (PAF). In contrast, about 65% of platelet serotonin is released by collagen or thrombin. In general, agents inducing more activation also elicit more platelet recruitment.²⁷

Thromboxane A₂ inhibits stimulated platelet adenylate cyclase, thereby acutely lowering platelet cyclic AMP. Thromboxane A₂ induces a rise in the concentration of ionized calcium in the platelet cytosol. Thromboxane is classified as an autacoid because it is rapidly converted to a biologically inactive hydration product (thromboxane B₂). Thromboxane A₂ is a vasoconstrictor and may serve to amplify the action of the platelet's other vasoconstrictor—serotonin.

If the thromboxane precursors (PGG₂ or PGH₂) are added to platelet-rich plasma, aggregation and serotonin release occur. A logical conclusion is that the platelets con-

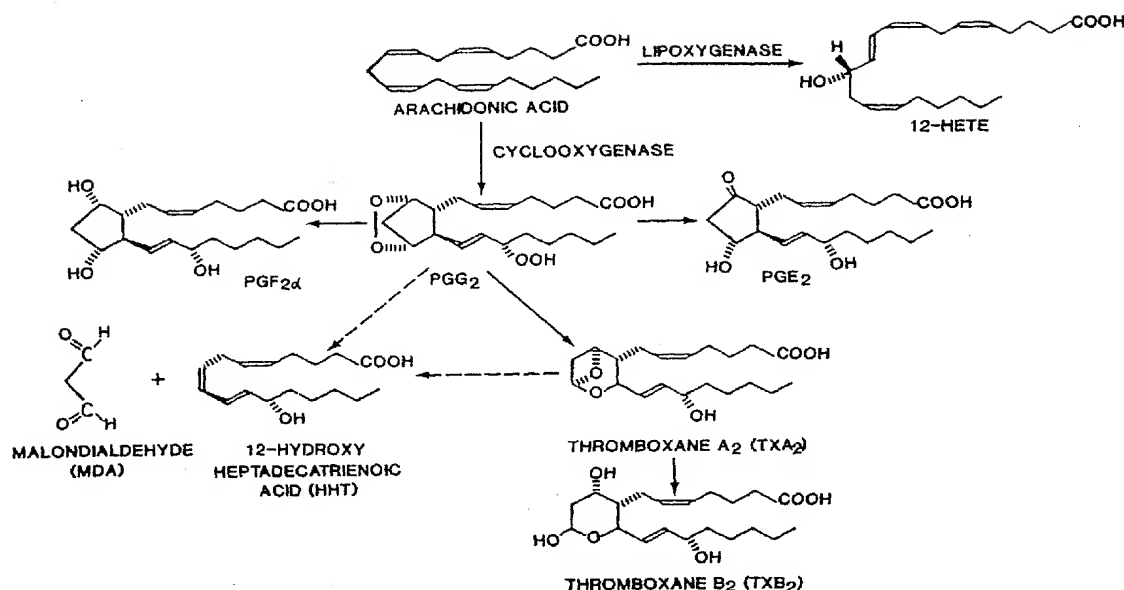


FIGURE 28-4 Enzymatic oxygenation of free arachidonate in platelets. A cytoplasmic 12-lipoxygenase can act on arachidonate to produce 12-HETE (upper right). This step is not inhibited by aspirin and will continue until free arachidonate is no longer available to the enzyme. The cyclooxygenase is particulate, and it oxygenates free arachidonate to prostaglandin G₂. The most important metabolite of PGG₂ is thromboxane A₂. This eicosanoid, which is rapidly converted to an inactive metabolite, thromboxane B₂, is a vasoconstrictor and induces platelet aggregation, accompanied by approximately 28% serotonin release. Thromboxane A₂ acts by inhibiting platelet adenylate cyclase activity, thereby lowering platelet cyclic AMP and reducing its inhibition of free calcium mobilization. Two other compounds are formed from PGG₂, neither of which has been functionally characterized. One is HHT (now known as HHTrE, a 17-carbon compound). Although HHTrE has mild chemotactic properties, as do other hydroxy acids, it does not have any functional implications known at the present time. The additional three carbons from HHTrE account for malondialdehyde (MDA), which also defies functional classification.

vert the endoperoxide to thromboxane, except that nonmetabolizable endoperoxide analogues such as U46619 also induce platelet aggregation.^{17,18} Therefore, endoperoxides themselves may serve as platelet agonists. In any case, both endoperoxides and thromboxane induce their biologic effects by way of release of adenosine diphosphate, which is the final common pathway in most platelet aggregation responses. This mechanism can be demonstrated in vitro by enzymatic removal of ADP from the system or by the use of agents that block ADP receptors. A full aggregation response cannot be elicited under these circumstances.

Several unanswered questions concern the function of biochemical components of the cyclooxygenase pathway. For example, the compound 12-hydroxy-5,8,10-heptadecatrienoic acid (HHTrE) is a by-product of endoperoxide formation. This 17-carbon compound, in common with other hydroxy acids, is mildly chemotactic, but there is no information on its role in platelet function. Interestingly, the remaining three carbons deleted from the endoperoxide during HHTrE formation can be accounted for by malondialdehyde (MDA). This substance is potentially toxic

because it crosslinks proteins. Why it is formed during a presumably beneficial process such as platelet aggregation has never been comprehended (see Fig. 28-4).

Prostacyclin

In sharp contrast to platelets, blood vessels do not contain a thromboxane synthase but rather contain an enzyme that transforms endoperoxides into prostacyclin. Endothelium also produces PGE₂, PGD₂, and PGF_{2α}. The autacoid, prostacyclin, induces marked vasodilation and inhibition of platelet function. The latter occurs by way of elevation of platelet cyclic AMP through stimulation of adenylate cyclase. Elevated levels of cyclic AMP in platelets lead to calcium reuptake in the dense tubular system. This reuptake is controlled by a calcium pump and catalyzed by a calcium ATPase. Prostacyclin was identified much later than other eicosanoids because of its extreme lability in aqueous media below pH 7, which results in formation of a biologically

inactive end product—6-keto-PGF_{1 α} —in a nonenzymatic manner. The biologic effects of PGI₂ were initially identified in bioassay systems,⁷ and its biochemical properties identified later.

It was proposed that fluidity of blood could be controlled by a "balance" between prostacyclin and thromboxane production, because their biologic effects seemed to be diametrically opposed. This concept was important for the therapeutic use of aspirin in ischemic vascular diseases. Endothelial cells can resynthesize prostacyclin soon after aspirin exposure, whereas platelets are unable to resynthesize thromboxane. A concern was that after aspirin ingestion, prostacyclin production would be completely lost for a period, during which its protective effect would be absent. However, there have been successful clinical trials of the antithrombotic effects of aspirin in which production of prostacyclin was virtually eliminated.²⁸ Thus, the presumed protective role of prostacyclin is not essential for successful therapy. In addition, two antithrombotic systems, both of which are insensitive to aspirin, have received recent attention—the cell-associated ADPase system on endothelial cells²⁹ and the endothelium-dependent relaxing factor (EDRF/NO).³⁰ These have necessitated rethinking of older concepts concerning control of platelet reactivity by endothelial cells.³¹

Platelet and Neutrophil Lipoxygenase Pathways

Platelet eicosanoid research in the past has focused mainly on the cyclooxygenase product—thromboxane—because it has direct functional and clinical implications. The platelet lipoxygenase, also involved in arachidonate oxygenation, is a soluble enzyme that catalyzes formation of 12L-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE). Released platelet arachidonate is initially converted to a hydroperoxy precursor, 12-HPETE, and then a peroxidase converts 12-HPETE to 12-HETE. Production of 12-HETE is abundant and continues until the free arachidonate is consumed. The molecule is mildly chemotactic but might be biologically active because of the large quantities produced.

An interesting metabolic relation exists between platelets and neutrophils—at least *in vitro*. The neutrophil metabolizes released platelet 12-HETE to dihydroxy acids, the nature of which depends on whether the neutrophil itself has been activated.^{13,16,20,32}

On the evolutionary scale, cytoplasmic lipoxygenases appeared before the cyclooxygenases did. In plants, oxygenation of linoleic acid is catalyzed by a lipoxygenase. Recent information on amino acid sequences indicates that the 5-lipoxygenase from rat tissues resembles the soybean 15-lipoxygenase.²³ Platelet lipoxygenases were the first to be described in mammalian cells.¹⁷ Now we know that lipoxygenase activities specific for other regions on the arachidonate molecule exist (positions 5, 11, and 15). These lipoxygenases catalyze the formation of autacoids involved

in the inflammatory response and host defense mechanisms in general.³³

Studies of the 5-lipoxygenase pathway in inflammatory cells were initially carried out in 1976 by Borgeat and Samuelsson.³⁴ Leukocytes were prelabeled with arachidonate and maximally activated with the calcium ionophore A23187. Neutrophils, in particular, do not have a cyclooxygenase pathway but do produce 5-hydroxyeicosatetraenoic acid (5-HETE) and 5(S),12(R)-DiHETE or leukotriene B₄ (LTB₄) (see Fig. 28-3). This dihydroxyeicosatetraenoic acid is the most powerful proinflammatory substance yet described. Its chemotactic and chemokinetic properties are far more potent than those of any other eicosanoid.

The 5-lipoxygenase pathway in leukocytes also accounts for a biologic activity that was known for many years but never characterized. This is the slow-reacting substance of anaphylaxis (SRS-A), which produces a prolonged contractile response in smooth muscle. The activity may be responsible for reactions of immediate hypersensitivity, asthma, and similar long-lasting proinflammatory phenomena.³³ SRS-A consists of a parent compound LTC₄ plus its metabolites LTD₄ and LTE₄. LTC₄ is formed by conjugation of glutathione to the initial metabolite LTA₄ by glutathione-S-transferase. Therefore, LTC₄ contains cysteine, glycine, and glutamic acid. The two other sulfur-containing leukotrienes LTD₄ and LTE₄ contain cysteine plus glycine, and cysteine alone, respectively. Formation of LTD₄ and LTE₄ is catalyzed sequentially by monocyte-macrophage gamma-glutamyl transpeptidase and dipeptidase (see Fig. 28-3).

Leukotriene B₄ may play a role in thrombosis by promotion of neutrophil adhesion to vascular endothelium. It is also a complete secretagogue and induces superoxide and hydrogen peroxide generation. Leukotrienes C₄, D₄, and E₄ induce bronchoconstriction, increase the permeability of postcapillary venules, and stimulate mucous secretion. Clinical studies involving inhibitors of the cysteinyl leukotrienes for treatment of asthma are currently under way.³⁵

The presence of a cysteine residue at the sixth carbon of the leukotriene molecule confers the ability to contract smooth muscle. Recent studies of transcellular metabolism between platelets and neutrophils by Maclouf and Murphy have particular clinical relevance.²⁵ It was demonstrated that platelets contain a glutathione-S-transferase that can convert neutrophil-derived LTA₄ to LTC₄. As already mentioned, leukotriene metabolism is aspirin-insensitive. This may explain why patients with unstable angina continue to have symptoms of myocardial ischemia while being treated with aspirin²⁶; although the platelets are not producing thromboxane, they can synthesize LTC₄ from activated neutrophils, which in turn induces coronary vasoconstriction.²⁶

The lipoxins, a novel group of proinflammatory eicosanoids discovered by Serhan, are formed by the action of 5- and 15-lipoxygenases.³⁶ Lipoxin A contracts pulmonary cells, and both lipoxin A and lipoxin B can inhibit the cytotoxicity of natural killer cells. Transcellular metabolism between neutrophil lipoxins and platelets has also been demonstrated.³⁷

Eicosanoid Receptors

Cell-surface receptors for lipids such as eicosanoids are very difficult to isolate. This means that specific reconstitution experiments with other cell proteins are difficult to conduct.²³ In all likelihood, eicosanoids do interact with cells by way of surface receptors. If we assume these to be analogous to adrenergic receptors, we would expect to find direct coupling to guanine regulatory proteins. For example, in the kidney it is known that PGE_2 interacts with two receptors: one is stimulatory in nature and coupled to G_s . In this mechanism, adenylate cyclase is activated (similar to the β -adrenergic receptor). PGE_2 also acts by way of an inhibitory receptor that is coupled to G_i . This would inhibit elevations of adenylate cyclase (similar to α_2 -adrenergic receptors). Thus, eicosanoids interact with specific receptors that are coupled to G proteins. Receptor occupancy in the presence of guanosine triphosphate eventually results in interactions with adenylate cyclase, phospholipase C, or calcium channels. Changes in the concentration of second messengers such as cyclic AMP, inositol phosphates, diacylglycerol, or calcium then occur.^{38,39}

Platelet Phosphoinositides

Although phosphatidylinositol (PI) constitutes only 5% of lipid phosphorus in human platelets, the metabolism of this phospholipid plays an important role in stimulus-response coupling. This was initially termed the "phosphoinositide effect" by Hanahan and Nelson in 1984.⁴⁰ Ac-

tivation of many cell types is accompanied by enhanced phospholipid turnover, especially of PI and phosphatidic acid (PA), which is accompanied by elevation of intracellular calcium. The sequential phosphorylation of PI gives rise to phosphorylated derivatives—PI-4-phosphate (PIP) and PI-4,5-bisphosphate (PIP_2) (Fig. 28-5). PIP_2 is rapidly degraded after cell stimulation. The resulting products are 1,2-diacylglycerol and inositol 1,4,5-trisphosphate (IP_3). Diacylglycerol activates cytoplasmic protein kinase C. Upon activation, protein kinase C binds to the cell membrane and increases its affinity for calcium. In platelets, activation of protein kinase C is accompanied by phosphorylation of several proteins, including one of 20 kD and another of 40 kD. The diacylglycerol (arachidonyl-stearyl) becomes phosphorylated to phosphatidic acid.⁴¹ The PA is subsequently metabolized to cytidine diphosphate-diacylglycerol and then back to PI (completing the "PI cycle"). The IP_3 that form by way of action of phospholipase C on PIP_2 is a mediator of calcium release from the dense tubular system of platelets.^{42,43} Thus, a single cycle of PI turnover can generate two separate intracellular mediator molecules—diacylglycerol and IP_3 . Three molecules of adenosine triphosphate are consumed in the process.

Turnover of phosphoinositides provides a link between platelet agonists and transduction of signals that they evoke. Thus, an agent such as thrombin, through its receptor,⁴⁴ can independently elicit two pathways of response induction: activation of protein kinase C and mobilization of calcium.⁴⁵ The system is bypassed when platelets are exposed to ionophore A23187 (which mobilizes calcium directly) or to phorbol myristate acetate (which activates protein kinase C directly) (see Fig. 28-5).

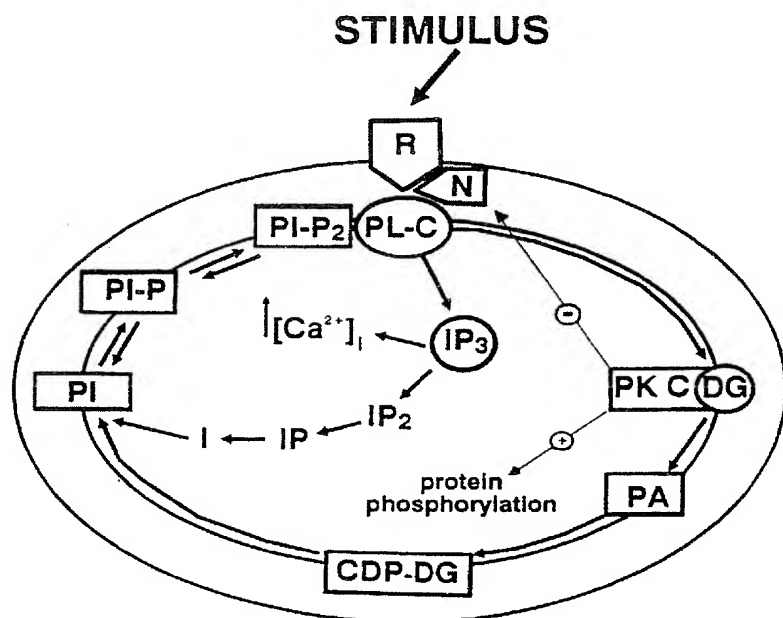


FIGURE 28-5 Phosphoinositide metabolism in platelet stimulus-response coupling. In unstimulated platelets, there is an equilibrium between PI, PI-P, and PI-P_2 . A platelet agonist will activate phospholipase C (PLC) by way of stimulation of a regulatory guanine nucleotide-binding protein (N). This results in formation of two second messengers from PI-P_2 : inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DG). IP_3 releases calcium intracellularly from the dense tubular system. Increases in intracellular calcium lead to activation of several calcium-dependent enzymes.

Cell-Cell Interactions and Transcellular Metabolism in the Eicosanoid System

Eicosanoids represent a class of labile autacoids that exert activating or inhibiting activities in the microenvironment of cells that synthesize them.³ During hemostasis, thrombosis, and the inflammatory process in general, multiple cell types are brought into close proximity. This increases the opportunity for metabolic interactions between precursors, intermediates, and end products of different cells that are capable of metabolizing these molecules. Research^{9,25} has led to a classification of biochemical reactions between more than one cell type involving components of the eicosanoid system (Table 28-6). Type I: A common precursor is shared by two or more stimulated cells in close proximity. In addition to synthesizing its own eicosanoid precursor, a cell can also acquire this precursor if it is released or present on the surface of a cell in close proximity (type IA). This results in production of more end product than the cell could have synthesized alone. An illustration of type IA is the use of platelet-derived endoperoxides for production of prostacyclin by endothelial cells that have been pretreated with aspirin. Aspirin-treating the endothelial cells verifies that it was the platelet endoperoxide that was used for prostacyclin production. When endothelial cells stimulated in the presence of platelets are not treated with aspirin, more prostacyclin is produced than could have been synthesized by endothelial cells alone. This effect results from endothelial production of PGI₂ by endogenous mechanisms in addition to utilization of platelet endoperoxides and released platelet arachidonate.⁹

In cell-cell interaction type IB, a particular cell cannot produce a precursor endogenously but possesses enzymatic mechanisms for further processing this precursor if it is available from a stimulated cell in proximity. This is exemplified by erythrocytes that cannot produce leukotriene A₄ but can process it to leukotriene B₄ if leukotriene A₄ is made available by a stimulated neutrophil in the microenvironment.⁴⁶ Another example is the endothelial cell, which ordinarily cannot synthesize leukotriene C₄ but will do so if

it acquires leukotriene A₄ from a stimulated neutrophil.⁴⁷ In addition, platelets contain glutathione-S-transferase, and if they can acquire leukotriene A₄ from a stimulated neutrophil, they will synthesize LTC₄.^{25,26}

Type II: These are cell-cell interactions that involve transformation of an eicosanoid originating from one cell into another compound that neither cell is capable of synthesizing alone. These reactions may be divided into two classes, based upon whether one or both cells have been activated. In type IIA, both cells have been exposed to a common stimulus, and they give rise to a new metabolite. This can be demonstrated by stimulating platelets and neutrophils with the common agonist ionophore A23187. Under these conditions, 12-HETE released by platelets will be metabolized by the activated neutrophil 5-lipoxygenase to 5(S),12(S)-DiHETE. In addition, this cell-cell interaction reduces the quantity of the neutrophil product 5(S),12(R)-DiHETE (LTB₄) that will be formed.

The type IIB cell-cell interaction can be demonstrated when one component of a dual-cell system is activated and the other is not. If a platelet-neutrophil suspension is stimulated with platelet agonists that do not activate neutrophil eicosanoid metabolism, such as thrombin or collagen, the compound 12,20-DiHETE is synthesized.¹⁶ This eicosanoid cannot be produced by platelets or neutrophils alone. The biochemical background of this interaction is a cytochrome P-450 omega-hydroxylase in the neutrophil that converts platelet 12-HETE to neutrophil-derived 12,20-DiHETE. The neutrophil then further metabolizes 12,20-DiHETE to 12-HETE-1,20-dioic acid by way of a dehydrogenase mechanism.¹³

The type III cell-cell interaction is characterized by activation or inhibition of one cell type by an eicosanoid originating in a neighboring cell. Thus, the spasmogenic leukotrienes, C₄, D₄, and E₄, can act as agonists for induction of cyclooxygenase metabolism in a tissue or organ in proximity. Infusion of LTC₄ or LTD₄ can constrict guinea pig trachea and the constriction is inhibited by indomethacin.³

Control of Platelet Reactivity by Intact Erythrocytes

Participation of erythrocytes in hemostasis and thrombosis is an accepted postulate, but experimental evidence has usually been fragmentary. The bleeding time is prolonged in anemic patients, and this can be corrected by normalization of the hematocrit. Traditional explanations include liberation of proaggregatory substances such as ADP from damaged or fragmented erythrocytes.⁴⁸ Recent studies have indicated that enhancement of platelet reactivity is a functional property of intact, metabolically active erythrocytes.²⁷ It is possible to study cell-cell interactions immediately after platelet activation in an experimental system (Fig. 28-6). One finds that a major consequence of platelet activation is release of components of intracellular gran-

TABLE 28-6 Metabolic Interactions Between Precursors, Intermediates, and End Products of the Eicosanoid Pathway in Different Cells

Type I

Different cells can biochemically process a common precursor.

Type II

Eicosanoids from one cell can be converted into a new metabolite that neither of the combined cells can produce alone.

Type III

An eicosanoid, precursor, or intermediate released from one cell can act as an agonist or inhibitor for another.

Generating System (Activation)

Assay System (Recruitment)

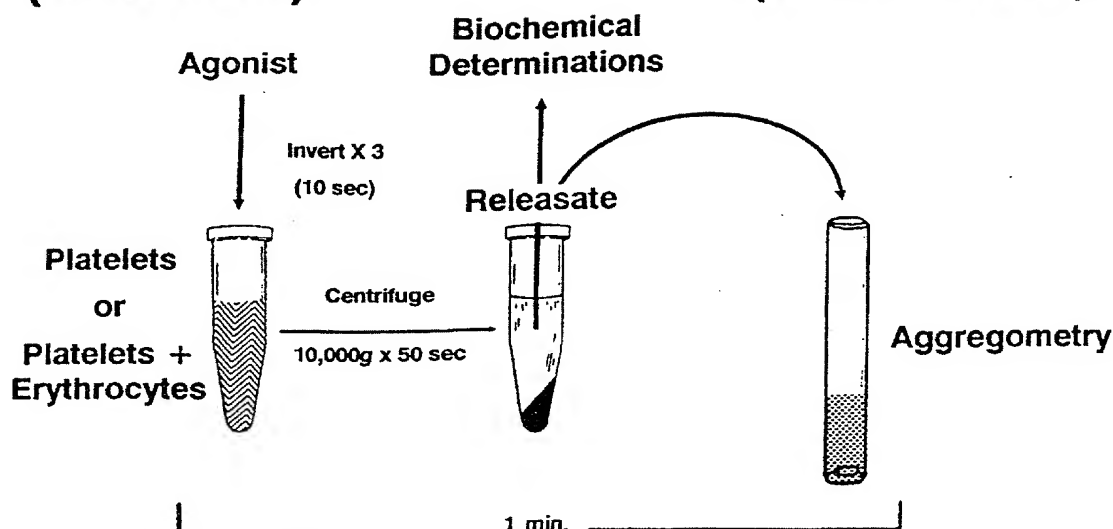


FIGURE 28-6 System for separate evaluation of activation and recruitment. Agonists are added to the generating system, which can activate platelets or combine suspensions of platelets and other cells. In this instance, the erythrocyte is shown. The cell-free releasate obtained after centrifugation of the generation system is transferred to the assay system (platelet-rich plasma) for assessment of proaggregatory activity (recruitment phase). The material can also be transferred for biochemical evaluation of platelet activation. (Courtesy of The Journal of Clinical Investigation 87:571–580, 1991)²⁷

ules, which in turn activate other platelets and also interact with other cells in the microenvironment during the “recruitment phase” of hemostasis or thrombosis.

A system such as that shown in Figure 28-6 allows independent evaluation of platelet activation and platelet recruitment. Platelets, or combined suspensions of platelets with erythrocytes or neutrophils, can be stimulated in the generating system, and the separated cell-free releasate can be studied biochemically and biologically. The biologic response is implemented by adding a portion of the releasate to human platelet-rich plasma (assay system). The releasate acts as an agonist for evaluation of the recruitment phase. Erythrocytes increase platelet serotonin release in the generating system, even after aspirin treatment, enzymatic removal of adenosine diphosphate, or protease inhibition. Erythrocytes also increase platelet arachidonate release and eicosanoid formation by way of both cyclooxygenase and lipoxygenase pathways.

Principles of Thromboregulation

Current concepts of the pathogenesis of coronary and cerebrovascular thrombosis involve two main phenomena: (1) ulceration or fissuring of the fibrous encasement of athero-

sclerotic plaques, which culminates in platelet adhesion, activation, and recruitment^{31,49–51}; and (2) shear-induced platelet activation at sites of stenotic lesions that also represent localized areas of disturbed blood flow.⁵² These aberrations overcome natural defense mechanisms (thromboregulators) and result in a multicellular cascade leading to thrombin formation and vessel occlusion. These aggressive prothrombotic events may not have been considered multicellular previously, but such a concept may explain why therapeutic modalities such as aspirin, which does not prevent the initial phases of platelet aggregation, have provided only modest protection against thrombotic disorders. A critical appraisal of clinical trials of aspirin suggests that less than 50% of the patients have an increase in survival.²⁴ Combinations of aspirin and other agents may be more effective than aspirin alone because the compound whose generation aspirin inhibits—thromboxane A_2 —is not a strong platelet agonist in vitro (thromboxane evokes only 28% serotonin release in platelets). A strategy for inhibition of vascular occlusion that takes a more global multicellular approach may be more complicated to develop than a unicellular concept, but it may have more potential and greater safety.

Thrombosis and atherosclerosis are multicellular processes, the pathogenesis of which is governed by cell proximity, contact, and activation. Thrombotic/hemostatic and inflammatory events are biochemically linked as compo-

nents of host defense mechanisms. Biochemical and functional dissection of these interactions in vitro has yielded new information relative to the pathogenesis of thrombosis and the inflammatory response.³¹ In the future, in addition to controlling eicosanoid metabolism between cells, enhancement of endothelial cell ADPase activity may be protective. Furthermore, there may be pharmacologic maneuvers, such as administration of the precursor arginine, that can increase production of EDRF/NO. Amplification of neutrophil inhibition of platelet reactivity could also serve as an antithrombotic modality. Future goals should include attempts at therapeutic enhancement of cell-cell interactions that have antithrombotic potential and inhibition of interactions that are prothrombotic, such as erythrocyte amplification of platelet reactivity. This may be the approach of choice for the 1990s.

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